Glycoconjugate Journal (1993) **10**:34–39

Direct demonstration of heterogeneous, sulfated *O*-linked carbohydrate chains on an endothelial ligand for L-selectin

YASUYUKI IMAI* and STEVEN D. ROSEN‡
Department of Anatomy and Program in Immunology, University of California, San Francisco, CA 94143-0452, USA
Received 6 October 1992

We have previously identified endothelial ligands for L-selectin as sialylated, fucosylated and sulfated glycoproteins of approximately 50 kDa and 90 kDa (Sgp50 and Sgp90). In this report, we use the beta elimination reaction to demonstrate directly the presence of sulfated *O*-linked sugar chains on one of these ligands, after metabolic labeling with radiolabeled sulfate or fucose. All of the sulfated and the majority of the fucosylated *O*-linked sugar chains were shown to be sialylated by affinity chromatography on a Limax agglutinin column. Analyses by anion exchange and gel permeation chromatography revealed a complexity of sugar chains, which were heterogeneous both in charge and size. Charged groups other than sialic acid appeared to exert a predominant influence on the total charge of the sugar chains. The probable existence of a varying number of sulfate modifications per sugar chain is discussed.

Keywords: L-selectin, ligand, GlyCAM-1, sialic acid, sulfate, fucose

Introduction

L-Selectin is a lectin-like receptor on lymphocytes and other leukocytes, and is a member of the selectin family of cell–cell adhesion molecules [1]. Members of this family share a distinctive domain organization of protein motifs, consisting of an amino-terminal calcium dependent (C-type) lectin domain, a single epidermal growth factor (EGF) motif, two or more numbers of complement-regulatory-like (CR-like) domains, followed by a transmembrane domain and a short cytoplasmic tail [2]. L-Selectin was first identified on lymphocytes as a lymph node homing receptor [3], involved in the initial attachment of lymphocytes to the specialized endothelial cells of high endothelial venules (HEV) within lymph nodes (reviewed in [4]).

We have identified and characterized the biological ligands on lymph node HEV as sulfated, fucosylated, and sialylated glycoproteins [5], termed Sgp50 and Sgp90, by the use of a soluble recombinant L-selectin-immunoglobulin chimera (LEC-IgG) molecule [6] as an affinity matrix. The nature of the interaction between LEC-IgG and the Sgp's is consistent with the participation of the C-type lectin domain of L-selectin. The interaction is blocked by mAbs known to interfere with lymphocyte adhesion to lymph node HEV [3, 5, 7, 8]. The interaction is dependent on the presence of sialic acid on the Sgp's [5, 7], consistent with the functional requirement of sialic acid on the HEV ligands [9]. Recently, one of the Sgp's, Sgp50 or GlyCAM-1, has been cloned, and its amino acid sequence predicts the presence of mucin-like domains [10].

Although the sugar chains on the Sgp's are considered to be recognition targets of the C-type lectin domain of L-selectin, information about the sugar chains of the Sgp's has been very limited. Our initial study demonstrated the resistance of the sugar chains of the Sgp's to *N*-glycanase treatment [5], ruling out a major contribution of *N*-linked sugar chains. Subsequently, it was shown that treatment of Sgp50 with trifluoromethane sulfonic acid, a chemical procedure which releases both *N*-linked and *O*-linked sugar chains from protein backbones [11], reduced its apparent molecular weight by at least 20 000 (Imai and Rosen, unpublished result). Furthermore, trypsin fragments of Sgp50 retained the binding capacity for LEC-IgG, suggesting the role of carbohydrate in the interaction [7]. Consistent with these findings, the predicted protein sequence of Sgp50 (i.e. GlyCAM-1) contains two Ser/Thr rich regions (mucin-like domains) with the potential for the presentation of highly clustered *O*-linked chains [10]. Only one potential site for *N*-glycosylation is present in the cloned sequence. In the current study, we demonstrate that Sgp50 contains heterogeneous *O*-linked chains, which are variably modified with sulfate, fucose, and sialic acid.

* Present address: Department of Chemical Toxicology and Immunochemistry. The University of Tokyo, Tokyo 113, Japan.
* To whom correspondence should be addressed.

Materials and methods

Reagents

[^{35}S]Sodium sulfate and L-[5,6-^{3}H]fucose were purchased from ICN Radiochemicals (USA), [2-^{3}H]Mannose from NEN (USA), CHAPS from Boehringer-Mannheim Biochemicals (USA), Dowex 50W-X8 and Bio-Gel P-4 (200–400 mesh) from Bio-Rad (USA), Sephadex G-50, Sephadex G-25, QAE-Sepharose A25, CNBr-activated Sepharose 4B, and *N*-acetylneuraminic acid from Sigma (USA), *Limax flavus* agglutinin, *Arthrobacter ureafaciens* sialidase from Calbiochem-Behring Corp. (USA), ^{3}H labeled bi-sialylated, galactosylated bi-antennary alditol prepared from human fibrinogen from Oxford Glycosystems (USA), and laminari-oligosaccharides (a series of β(1-3)-linked glucose oligosaccharides from mono- to heptasaccharide) from Seikagaku America (USA). A recombinant mouse L-selectin-human IgG_1 chimera protein (LEC-IgG) was prepared as previously described [6] and was generously provided by Larry Lasky and Chris Fennie of Genentech Inc.

Preparation of oligosaccharides from radiolabeled Sgp ligands

Radiolabeled Sgp50 and Sgp90 were derived from a detergent extract of mouse lymph nodes labeled with sodium [^{35}S]sulfate or [^{2}H]fucose in organ culture for 4 h as described previously [5, 7]. The Sgp ligands were affinity isolated by means of immobilized LEC-IgG and concentrated in 10 mM CHAPS:0.15 M NaCl on a Centricon 30 (Amicon, USA). Since with both labels, Sgp50 was the predominant species (>95%), we will refer hereafter to the purified ligand as Sgp50. The Sgp50 was diluted by 1:10 in 0.5 ml water, and 0.5 ml 2 M $NaBH_4$ in 0.2 M NaOH was added. The mixture was incubated for 24 h at 45 °C to release the *O*-linked sugar chains [12]. After addition of acetic acid to stop the reaction, the reaction mixture was passed through a column of Dowex 50W-X8 (H^+ form, 3 ml volume) at 4 °C to remove sodium ions. The column was washed with 25 ml ice cold water and the flow-through fractions were evaporated and dissolved in methanol:1% acetic acid. Borate ion was removed by evaporation from methanol: acetic acid followed by evaporation from methanol [12]. The material was dissolved in 3 ml water and extracted with water-saturated butanol twice (3 ml each) to remove detergent. The water phase was recovered and evaporated and then fractionated on a column of Sephadex G-50 in 0.1 M pyridine acetate, pH 5.4.

Column chromatography

A Sephadex G-50 column (1 cm × 45 cm) was equilibrated either in 0.15 M NaCl or 0.1 M pyridine acetate, pH 5.4, and fractions (1.5 ml) were collected. Aliquots were counted by liquid scintillation. For the latter column, fractions with radioactivity were pooled, evaporated and subjected to gel filtration on a column of Bio-Gel P-4 (1 cm × 110 cm) equilibrated in 0.1 M pyridine acetate, pH 5.4. Fractions of 1 ml were collected, and aliquots were counted. For ion exchange chromatography, a sample was dissolved in 2 ml of 2 mM Tris-HCl, pH 9.0, and applied to a column of QAE-Sephadex A25 (0.8 cm × 4 cm) equilibrated in the same buffer. A step gradient was used for elution: steps of 0, 20, 70, 140, 200, 250, 400, 1000 mM NaCl in the Tris buffer with 6 × 2 ml fractions per step. Alternatively, a QAE-Sephadex A25 column (0.8 cm × 14.5 cm) was equilibrated in 25 mM KH_2PO_4, pH 4.0, according to methods of Green and Baenziger [13]. The applied sample was dissolved in 2.5 ml phosphate buffer. A linear gradient of 0–1200 mM NaCl (total volume of 150 ml) was used to elute the material. Limax agglutinin was coupled to CNBr-activated Sepharose 4B (2 mg per ml beads) according to the method of Miller [14]. A column of the Limax-Sepharose (0.5 cm × 5 cm) was equilibrated in Dulbecco's phosphate-buffered saline (PBS) containing 0.02% NaN_3 at 4 °C. A sample equilibrated in PBS was applied to the column and fractions of 0.47 ml were collected. The bound material was eluted with 50 mM *N*-acetylneuraminic acid in PBS. Samples were desalted on a Sephadex G-25 (1 cm × 45 cm) column equilibrated in water. All column chromatography except the Limax procedures was carried out at 22 °C.

Sialidase treatment

The labeled oligosaccharides were treated with 0.1 unit of *Arthrobacter* sialidase in 0.2 ml 0.1 M sodium acetate buffer, pH 5.0, for 3.5 h at 37 °C. The reaction was stopped by boiling for 5-min. This treatment was previously shown to remove all Limax-agglutinin reactive sialic acid from the intact ligand (unpublished observation). The sample was directly applied to a Bio-Gel P-4 column or diluted with 2.3 ml 25 mM KH_2PO_4, pH 4.0, and applied to a QAE-Sephadex column.

Results

Release of sulfate labeled O*-linked sugar chains from Sgp50*

We have previously shown that the Sgp glycoproteins were resistant to *N*-glycanase treatment [5] and that trifluoromethane sulfonic acid treatment reduced the molecular weight of Sgp50 (Imai and Rosen, unpublished). Based on these results, we attempted to isolate *O*-linked sugar chains from a Sgp preparation by use of the beta elimination reaction, which is a standard procedure for releasing *O*-linked sugar chains [12]. A ligand preparation containing mostly Sgp50 (>95%) was prepared from a detergent extract of [^{35}S]sulfate labeled mouse lymph nodes by affinity chromatography on LEC-IgG and was treated with alkaline borohydride. The released material was fractionated on a column of Sephadex G-50 equilibrated in 0.15 M NaCl (Fig. 1). The ^{35}S counts were totally included in this column, eluting as a broad peak around the elution position of ^{3}H

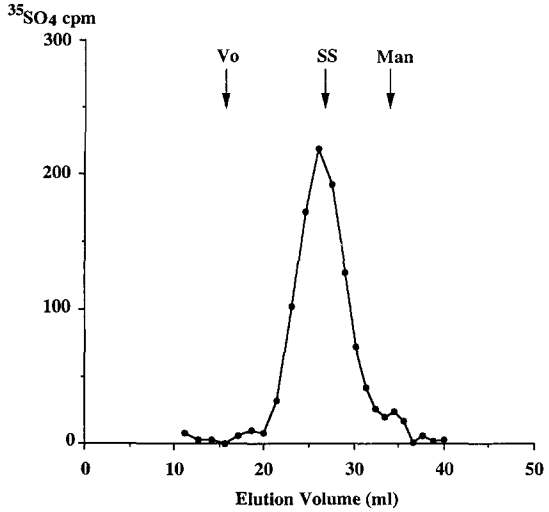

Figure 1. Sephadex G-50 profile of oligosaccharides released from [^{35}S]sulfate-labeled Sgp50 by alkaline-borohydride treatment. The column (1 cm × 45 cm) was equilibrated in 0.15 M NaCl, and an aliquot of each 1.5 ml fraction was counted. The column was calibrated with blue dextran (Vo), [^{3}H]-labeled bi-sialylated, galactosylated biantennary alditol (SS) and [^{3}H]mannose (Man). When the column was equilibrated in 0.1 M pyridine acetate buffer, pH 5.4, the profile was essentially the same. The recovery of the radioactivity from the ligand preparation was approximately 60%.

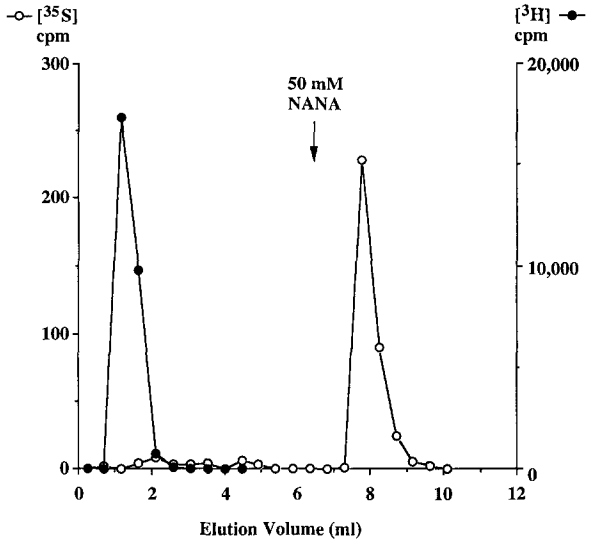

Figure 2. Limax agglutinin affinity chromatography of [^{35}S]sulfate labeled oligosaccharides of Sgp50. Released oligosaccharides (open circles) were purified on a column of Sephadex G-50 equilibrated in 0.1 M pyridine acetate, pH 5.4, before application onto the Limax column (0.5 cm × 5 cm) equilibrated in PBS at 4 °C. After extensive washing with PBS at a flow rate of 1.2 ml h^{-1}, *N*-acetylneuraminic acid (NANA) in PBS was introduced (shown by an arrow) to elute the bound materials. [^{3}H]Mannose was applied as a control (closed circles) and denotes the column volume.

labeled bisialylated, galactosylated biantennary alditol (denoted by 'SS' in Fig. 1). In order to facilitate further analysis, we employed the volatile buffer, 0.1 M pyridine acetate, pH 5.4, in this column, and obtained essentially the same elution profiles in repeated experiments (not shown). Fractions with radioactivity were pooled and evaporated for further analysis. We could not demonstrate direct binding of the released oligosaccharides to immobilized LEC-IgG (not shown), but trypsin fragments do bind [7], suggesting that clustering of *O*-linked chains may be essential for a significant interaction.

Sialylation of O*-linked sugar chains of Sgp50 ligand*

Sialic acid is present on the Sgp glycoprotein ligands and plays an essential role in their ligand function [5, 7]. It was therefore of interest to examine whether sialic acid was present on the isolated sulfated sugar chains. Sulfate labeled *O*-linked chains were purified on a Sephadex G-50 column and subjected to affinity chromatography on a column of Limax agglutinin, a sialic acid specific lectin of broad specificity [14]. As shown in Fig. 2, the material with ^{35}S counts was completely adsorbed on this column and was recovered by competitive elution with *N*-acetylneuraminic acid (abbreviated NANA in the figure). This result demonstrated that all sulfate *O*-linked chains are sialylated. Fucose is also present on the Sgp ligands [5], and our previous studies suggested the possible involvement of fucose in ligand activity [7, 15]. Thus, we labeled the Sgp50 with [^{3}H]fucose and isolated *O*-linked chains by the beta elimination. The released chains were purified on a Sephadex G-50 column, and the elution profile was essentially the same as in Fig. 1 (not shown). The radioactive fractions were pooled, evaporated, and subjected to affinity chromatography on the Limax column (Fig. 3). Material representing 80% of the total counts was adsorbed on the column and

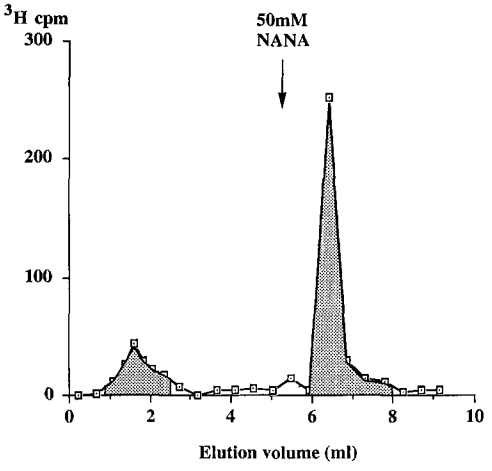

Figure 3. Limax agglutinin affinity chromatography of [^{3}H]fucose labeled oligosaccharides of Sgp50. Sephadex G-50 purified oligosaccharides were applied onto the Limax column as described in Fig. 2.

eluted with *N*-acetylneuraminic acid. This result demonstrated that the majority of fucosylated chains also have sialic acid.

Charge profile of sulfate or fucose labeled O-linked chains of Sgp50

The experiments described above established the relationship between sialic acid–sulfate and sialic acid–fucose in the *O*-linked chains of Sgp50. To study the relationship between sulfate and fucose, analysis on an anion exchange column was carried out. Sulfate labeled *O*-linked chains and fucose labeled *O*-linked chains (affinity purified on a Limax column) were analyzed in parallel on identical QAE-Sephadex columns. The results demonstrated considerable charge heterogeneity of these sugar chains (Fig. 4). Some of the fractions (e.g., 140, 200, 400, 1000 mM NaCl) showed coincidental peaks of sulfate and fucose. The contribution of sulfate counts relative to fucose counts was high in the fractions eluted with high salt concentration (400, 1000 mM), whereas the fucose counts were relatively enriched in the lower ionic strength fractions (70, 140, 200 mM). These results argue for heterogeneity in the representation of fucose and sulfate residues on the various *O*-linked chains.

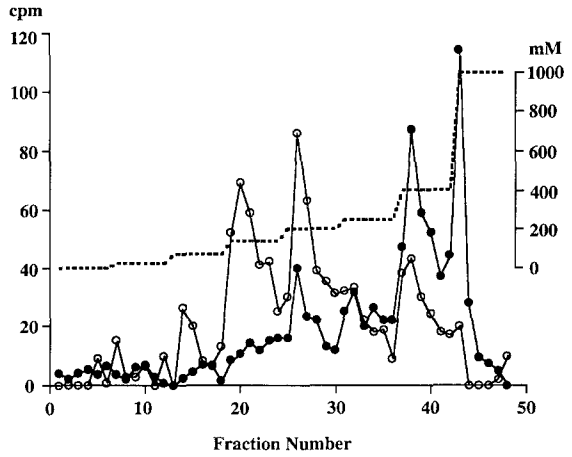

Figure 4. Anion exchange chromatogram of oligosaccharides of Sgp50. Two parallel columns of QAE-Sephadex A25 (0.8 cm × 4 cm) were equilibrated in 2 mM Tris-HCl, pH 9.0. A sample of [^{35}S]sulfate-labeled chains (closed circles) was applied to one column and a sample derived [^{3}H]fucose-labeled chains (open circles) was applied to the other. The bound material was eluted by a stepwise gradient of NaCl (0, 20, 70, 140, 200, 250, 400, 1000 mM) denoted by the dashed line. Fractions of 2 ml were collected and aliquots were counted. Prior to loading, the [^{35}S]sulfate-labeled and the [^{3}H]fucose-labeled oligosaccharides had been purified on a Sephadex G-50 column equilibrated in the pyridine acetate buffer. The [^{3}H]fucose-labeled chains had been further purified on a Limax agglutinin column with elution by *N*-acetylneuraminic acid and desalting on a column of Sephadex G-25.

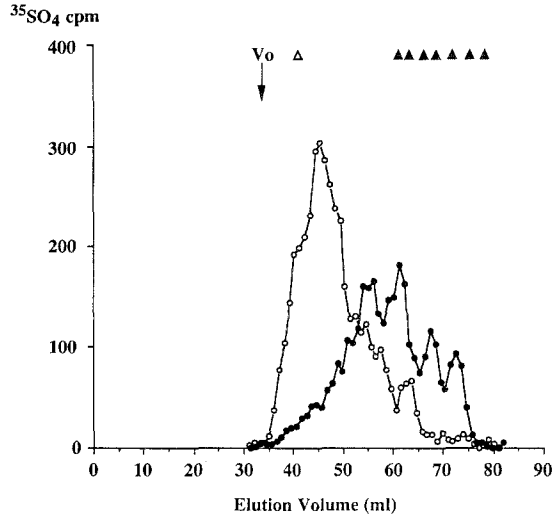

Figure 5. Size fractionation of oligosaccharides from Sgp50 with or without sialidase treatment. Oligosaccharides derived from [^{35}S]sulfate-labeled Sgp50 were purified on a Sephadex G-50 column. Sialidase-treated (closed circles) or untreated (open circles) oligosaccharides were analyzed on a column of Bio-Gel P-4 (1 cm × 110 cm) equilibrated in 0.1 M pyridine acetate buffer, pH 5.4. The open triangle shows the elution position of ^{3}H labeled bi-sialylated, galactosylated bi-antennary alditol ('SS'). The closed triangles represent mono- to heptasaccharides of the laminarioligosaccharide series (oligomers of Glc β1-3), which were detected by the phenol sulfuric acid reaction.

Size fractionation after desialylation

To gain more information on the size distribution of the *O*-linked chains, a 110 cm Bio-Gel P-4 column was used. Since, with this type of matrix, sialic acid is known to distort the elution profile [16], [^{35}S]sulfate-labeled *O*-linked chains were analyzed with or without treatment with sialidase. The untreated chains showed a broad asymmetrical peak starting at the elution position of 'SS' (Fig. 5). After sialidase treatment, six major distinguishable peaks and shoulders and several relatively minor peaks of higher molecular weight could be discriminated. Peaks corresponding to 3, 4.5, and 7 glucose units and three peaks/shoulders running larger than 7 glucose units were the predominent components observed.

Charge profile after desialylation

Since sialidase treatment dramatically changed the profile of sulfate labeled chains on the Bio-Gel P-4 column and the desialylated chains appeared to be small by their elution position, it seemed possible that the presence of multiple sialic acid substitutions (e.g., polysialic acid) might significantly contribute to the molecular mass of the *O*-linked chains. To test this possibility, we compared the profiles of sulfate labeled chains on an anion exchange column before and after sialidase treatment (Fig. 6). Sialidase generally shifted the elution positions toward the fractions with low

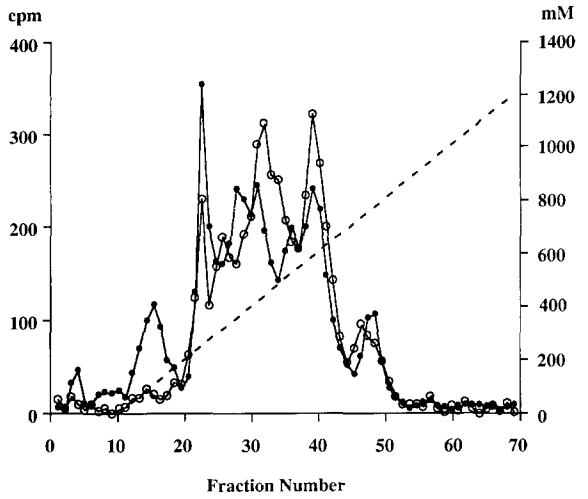

Figure 6. Anion exchange chromatogram of oligosaccharides from Sgp50 with or without sialidase treatment. Oligosaccharides derived from [^{35}S]sulfate-labeled Sgp50 were purified on a Sephadex G-50 column and treated with sialidase (closed circles) or buffer alone (open circles), and then analyzed on a QAE-Sephadex A25 column (0.8 cm × 14.5 cm) equilibrated in 25 mM KH_2PO_4, pH 4.0. The bound materials were eluted by a linear NaCl gradient (dashed line), which extended from 0 M to 1.2 M. Fractions of 2.5 ml were collected and counted.

salt concentration. However, there was no dramatic change in the elution profile, suggesting that sulfate groups or other charged groups not susceptible to sialidase removal were mainly responsible for the charge of the *O*-linked chains. The heterogeneity after desialylation also suggested the presence of chains with varying numbers of sulfate or other charged moieties.

Discussion

Our previous studies have characterized Sgp50 and Sgp90 as lymph node HEV-ligands for L-selectin. The binding of the C-type lectin domain of L-selectin to the sugar chains of the Sgp's was shown to be the basis of the interaction [5, 7, 8]. The biochemical and molecular characterization of the Sgp's indicated that *O*-linked chains represent the prevalent carbohydrate modification [5, 10]. In this report, we provide direct evidence for the presence of *O*-linked sugar chains on Sgp50, and present a preliminary characterization of these chains.

We subjected [^{35}S]sulfate- or [^{3}H]fucose-labeled Sgp50 to standard beta elimination, and found a broad peak of released oligosaccharides by analysis with Sephadex G-50. The elution position was close to that of [^{3}H]-labeled bi-sialylated, galactosylated bi-antennary alditol prepared from human fibrinogen, which consists of 11 sugars: sialic acid (2), galactose (2), *N*-acetylglucosamine (3), *N*-[^{3}H]-acetylglucosaminitol (1), and mannose (3). These results suggested the presence of relatively large *O*-linked sugar chains with a potential complexity that might be masked by the presence of charged residues such as sialic acid and sulfate.

We have previously shown an essential role of sialic acid for the function of the Sgp's [5, 7, 9]. To determine whether the sulfated or fucosylated *O*-linked chains of Sgp50 were sialylated, we employed affinity chromatography on a Limax agglutinin column. The results demonstrated that all of the sulfated chains and a majority (80%) of the fucosylated chains are sialylated.

To study the relationship between sulfated chains and fucosylated chains, we used anionic exchange chromatography. The sulfate-labeled chains (all possessing sialic acid) and the fucose-labeled sialylated chains (prepared by Limax affinity chromatography) were compared on an anion exchange column. In contrast to the elution pattern on Sephadex G-50, which showed a single broad peak, multiple peaks were recognized, indicating the presence of heterogeneous sugar chains with varying charge. Several peaks with sulfate and fucose labels ran coincidentally. Due to the limited resolution of this analysis, it is not possible to conclude that fucose and sulfate are simultaneously present on chains, although this possibility seems highly likely. Further analysis of isolated peaks is necessary to settle this issue.

Whereas the majority of fucose-labeled chains were eluted in the low salt (140–200 mM NaCl) fractions, the majority of sulfate labeled chains were eluted in the high salt (400–1000 mM NaCl) fractions, corresponding to the more highly charged fractions (Fig. 4). The salt concentration required for eluting the majority of sulfated chains was relatively high, according to the studies of Roux *et al.* [17]. This result may be explained by the presence of multiple sulfate residues on some of those sugar chains, which require high salt for elution. Green and Baenziger were able to fractionate sulfated chains from pituitary hormones by using a QAE-Sepharose column, equilibrated with a potassium phosphate gradient at pH 4.0 [13]. At this pH, both sulfate and sialic acid are ionized [13]. Under these conditions, 86% of the sulfated *O*-linked chains derived from Sgp50 remained adsorbed on the column at 250 mM KH_2PO_4 (data not shown), the concentration at which sulfated sugar chains of the hormones were eluted [13]. The disialylated standard ('SS') eluted at 80 mM KH_2PO_4 concentration. This result is consistent with the possibility of sugar chains that are multiply substituted with sulfates or other charged moieties.

To analyze the size distribution of the sugar chains in more detail, a gel filtration analysis on Bio-Gel P-4 was carried out. Since sugar chains with sialic acid substitutions are known to behave anomalously on Bio-Gel columns [16, 18], we performed the analysis before and after desialylation. With sialic acid present, the sugar chains were distributed in an asymmetrical broad peak. After sialidase treatment, the sugar chains were resolved into a complex

pattern of at least six major peaks and shoulders, reminiscent of the pattern obtained in the ion exchange analysis. The gel filtration chromatogram was far more complex than that reported for the *O*-linked sugar chains of a rat mammary adenocarcinoma mucin [18]. Unexpectedly, the apparent molecular weight of the smallest chain approximated a three glucose unit. Estimating the size of a single amino sugar to be two glucose units [16], the smallest core would be estimated to be as small as disaccharide. However, we found that the presence of sulfate on carbohydrate significantly modified its behavior on Bio-Gel P-4, as indicated by the observation that the elution position of glucose 6-sulfate (88 ml) was significantly later than that of glucose (77 ml). Thus, it is likely that the molecular weights of the sulfated *O*-linked chains (after desialylation) are actually substantially larger than the values obtained by comparison with the neutral standards. Desulfation as well as desialylation may be required to establish accurate values for the molecular weights of the core oligosaccharides.

The apparent low molecular weight of the desialylated *O*-linked chains raised the additional possibility that multiple sialic acid residues (e.g., polysialic acid) might contribute significantly to the molecular mass of the *O*-linked chains. To examine this possibility, elution patterns of the chains on an anion exchange chromatography were compared with or without sialidase treatment. The pH of the column buffer was chosen so that both sulfate and sialic acid residues were charged. Despite the fact that sialidase treatment significantly altered the Bio-Gel P-4 profile, the elution pattern on an ion exchange resin was only moderately shifted toward the low salt fractions, with no evidence for the disappearance of the highly charged species. This result suggests that sulfate groups (or other charged moieties not associated with sialic acid moieties) are mainly responsible for the charge of the *O*-linked chains, and that a contribution from multiple sialic acid residues such as polysialic acid is unlikely. In addition, the existence of several peaks after sialidase treatment confirms the presence of sugar chains with a varying number of charged groups (e.g., sulfates).

Acknowledgements

We thank Larry Lasky and Chris Fennie of Genentech Inc. for the gift of LEC-IgG. The research was supported by grants from NIH and Genentech, Inc. to SDR.

References

1. Bevilacqua M, Butcher E, Furie B, Furie B, Gallatin M, Gimbrone M, Harlan J, Kishimoto K, Lasky L, McEver R, Paulson J, Rosen S, Seed B, Siegelman M, Springer T, Stoolman L, Tedder T, Varki A, Wagner D, Weissman I, Zimmerman G (1991) *Cell* **67**:233.
2. Stoolman LM (1989) *Cell* **56**:907–10.
3. Gallatin WM, Weissman IL, Butcher EC (1983) *Nature* **303**:30–34.
4. Lasky LA, Rosen SD (1993) In *Inflammation, Basic Principles and Clinical Correlates*, 2nd Edition (Gallin JL, Goldstein IM and Snyderman R, eds). New York: Raven Press.
5. Imai Y, Singer MS, Fennie C, Lasky LA, Rosen SD (1991) *J Cell Biol* **113**:1213–21.
6. Watson SR, Imai Y, Fennie C, Geoffroy JS, Rosen SD, Lasky LA (1990) *J Cell Biol* **110**:2221–29.
7. Imai Y, Lasky LA, Rosen SD (1992) *Glycobiology* **2**:373–81.
8. Bowen B, Fennie C, Lasky LA (1990) *J Cell Biol* **110**:147–53.
9. Rosen SD, Singer MS, Yednock TA, Stoolman LM (1985) *Science* **228**:1005–7.
10. Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gilett N, Watson SR, Rosen SD (1992) *Cell* **69**:927–38.
11. Sojar HT, Bahl OP (1987) *Methods Enzymol* **138**:341–50.
12. Fukuda M (1989) *Methods Enzymol* **179**:17–29.
13. Green ED, Baenziger JU (1986) *Anal Biochem* **158**:42–9.
14. Miller RL (1987) *Methods Enzymol* **138**:527–36.
15. Imai Y, True DD, Singer MS, Rosen SD (1990) *J Cell Biol* **111**:1225–32.
16. Yamashita K, Mizuochi T, Kobata A (1982) *Methods Enzymol* **83**:105–26.
17. Roux L, Holojda S, Sundblad G, Freeze HH, Varki A (1988) *J Biol Chem* **263**:8879–89.
18. Hull SR, Carraway KL (1989) *J Cellular Biochem* **40**: 67–81.